Lipid Modification of Proteins through Sortase-Catalyzed Transpeptidation

John M. Antos, Gwenn M. Miller, Gijsbert M. Grotenbreg and Hidde L. Ploegh

Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, Massachusetts, 02142

Supporting Information

Contents:

I. Materials and Instrumentation

Unless otherwise noted, all chemicals were obtained from commercial sources and used without further purification. Rink amide resin (100-200 mesh, 0.7 mmol/g) was obtained from Advanced Chemtech. Fmoc-Gly-OH, Fmoc-Lys(Mtt)-OH, and Fmoc-Trp(Boc)-OH were obtained from EMD Biosciences/Novabiochem. Triglycine peptide was purchased from Sigma (G1377). Water used in biological procedures or as a reaction solvent was purified using a MilliQ purification system (Millipore). DriSolv® anhydrous CH_2Cl_2 and DriSolv® anhydrous MeCN were purchased from EMD Chemicals. Redistilled, anhydrous *N,N′*-diisopropylethylamine (DIPEA) was obtained from Sigma-Aldrich.

NMR. ^1H and ^{13}C spectra were measured with a Bruker AVANCE-400 (400 MHz) spectrometer at the MIT Department of Chemistry Instrumentation Facility. ^1H NMR chemical shifts are reported as δ in units of parts per million (ppm) relative to chloroform-*d* (δ 7.24, singlet). Multiplicities are reported as follows: s (singlet), d (doublet), or m (multiplet). Coupling constants are reported as a *J* value in Hertz (Hz). The number of protons (n) for a given resonance is indicated as nH, and is based on spectral integration values. ^{13}C NMR chemical shifts are reported as δ in units of parts per million (ppm) relative to chloroform-*d* (δ 77.23, triplet).

Mass Spectrometry. Electrospray Ionization (ESI) mass spectra were obtained at the MIT Department of Chemistry Instrumentation Facility. Matrix assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on a MALDI micro MX™ system (Micromass® MS Technologies, USA). Samples were co-crystallized using a sinapinic acid solution (10 mg/mL in 70:30 MeCN:H_2O with 0.1% TFA). LC-ESI-MS analysis was performed using a Micromass LCT mass spectrometer (Micromass® MS Technologies, USA) and a Paradigm MG4 HPLC system equipped with a HTC PAL autosampler (Michrom BioResources, USA) and a Waters Symmetry 5 μm C8 column (2.1 x 50 mm, MeCN:H_2O (0.1% formic acid) gradient mobile phase, 150 μL/min).

HPLC/FPLC. HPLC purifications were achieved using an Agilent 1100 Series HPLC system equipped with a Waters Delta Pak 5 μm, 100 Å C4 column (3.9 x 150 mm,

MeCN:H_2O gradient mobile phase containing 0.1% trifluoroacetic acid, 1 mL/min), a Waters Delta Pak 15 μm, 100 Å C18 column (7.8 x 300 mm, 2-propanol:H_2O gradient mobile phase, 2 mL/min), or a Waters Cosmosil 5PE column (8 x 250 mm, 2-propanol:H_2O gradient mobile phase, 1 mL/min) as indicated below. Size exclusion chromatography was performed on a Pharmacia AKTA Purifier system equipped with a HiLoad 16/60 Superdex 75 column (Amersham).

UV-Vis Spectrocopy. UV-Vis spectroscopy was performed on a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA).

Flow Cytometry. Flow cytometry was performed on a LSR-II system (BD Biosciences).

Spinning Disc Confocal Microscopy. Fluorescence microscopy was performed on a Nikon spinning disc confocal microscope with MetaMorph 7 software. Multidimensional acquisition was used to collect images in the 488, 647, and phase contrast channels from the same focal plane.

II. Synthesis and Characterization of Lipidated Triglycine Nucleophiles

Resin-Bound Intermediate 1. A glass solid-phase reaction vessel containing a fritted glass filter and a Teflon stopcock was loaded with Rink amide resin (1.0 g, 0.70 mmol). The resin was first washed/swollen extensively with *N*-methyl-2-pyrrolidone (NMP) by gentle agitation with a wrist action shaker. The Fmoc protecting group was then removed by treatment with ~30 mL of 80:20 NMP/piperidine for 20 min at RT. The resin was washed with ~30 mL of NMP (3x, 3-5 min per wash). Amino acid building blocks were then coupled as follows: Fmoc-protected amino acid (1.75 mmol, 2.5 equivalents relative to initial resin loading), benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) (911 mg, 1.75 mmol), *N*-hydroxybenzotriazole (HOBt) (236 mg, 1.75 mmol), and *N,N′*-diisopropylethylamine (DIPEA) (914 μL, 5.25 mmol) were dissolved in NMP to a final volume of 8.75 mL (200 mM final concentration of amino acid building block). This solution was mixed until all reagents had dissolved, and then added to the deprotected Rink amide resin. Couplings were incubated for 16-48 h at

RT. The resin was then washed with ~30 mL of NMP (3x, 3-5 min per wash). The extent of coupling was assessed by Kaiser test. In the event that the coupling was incomplete, the above procedure was repeated. Fmoc removal was then achieved by exposing the resin to ~30 mL of 80:20 NMP/piperidine for 20 min at RT, followed by additional washing with ~30 mL of NMP (3x, 3-5 min per wash). Cycles of amino acid coupling and Fmoc deprotection were then repeated to complete the synthesis of **1**. The resin was then washed with ~ 30 mL of CH_2Cl_2 (5x, 3-5 min per wash) and dried.

1-ad, 1-C12, and 1-C14 (General Procedure). A 3.0 mL fritted polypropylene syringe equipped with a capped hypodermic needle was loaded with dry resin **1** (50 mg). The resin was washed with 2.5 mL of CH_2Cl_2 (3x, 3-5 min per wash). Next, the 4-methyltrityl (Mtt) protecting group was removed by treatment with 2.5 mL of 94:5:1 CH_2Cl_2/TIPS/TFA at RT (5x, 5 min each) followed by washing with 2.5 mL of NMP (3x, 3-5 min per wash). The resin was then treated with a solution of the appropriate carboxylic acid (R_1-COOH, 0.12 mmol), PyBOP (65 mg, 0.12 mmol), HOBt (16 mg, 0.12 mmol), and DIPEA (65 μL, 0.38 mmol) in 1.0 mL of NMP. The reaction was incubated at RT for 21 h followed by washing with 2.5 mL of NMP (3x, 3-5 min per wash). Fmoc removal was achieved by treatment with 2.5 mL of 80:20 NMP/piperidine for 20 min at RT followed by washing with 2.5 mL of NMP (3x, 3-5 min per wash) and 2.5 mL of CH_2Cl_2 (3x, 3-5 min per wash). The peptide was cleaved from the resin with 2.5 mL of 95:3:2 TFA/TIPS/H_2O (4x, ~30 min each) and the combined cleavage solutions were concentrated *in vacuo*. **1-ad** was then precipitated from ether and dried under vacuum. Crude **1-C12** and crude **1-C14** were washed with hexanes (1 mL, 2x) and then dried under vacuum. The identity of all peptides was confirmed by LC-ESI-MS analysis (Figure S1). Peptides were used without further purification and the yield was not determined.

1-C16. A 3.0 mL fritted polypropylene syringe equipped with a capped hypodermic needle was loaded with dry resin **1** (200 mg). The resin was washed with 2.5 mL of CH_2Cl_2 (3x, 3-5 min per wash). Next, the 4-methyltrityl (Mtt) protecting group was removed by treatment with 2.5 mL of 94:5:1 CH_2Cl_2/TIPS/TFA at RT (5x, 5 min each).

The resin was then washed with 2.5 mL of NMP (3x, 3-5 min per wash). The resin was then treated with a solution of palmitic acid (103 mg, 0.40 mmol), PyBOP (208 mg, 0.40 mmol), HOBt (54 mg, 0.4 mmol), and DIPEA (207 μL, 1.2 mmol) in 1.6 mL of NMP. The reaction was incubated at RT overnight. The resin was then washed with 2.5 mL of NMP (3x, 3-5 min per wash). Fmoc removal was achieved by treatment with 2.5 mL of 80:20 NMP/piperidine for 20 min at RT followed by washing with 2.5 mL of NMP (3x, 3-5 min per wash) and 2.5 mL of CH_2Cl_2 (3x, 3-5 min per wash). The peptide was cleaved from the resin with 2.5 mL of 95:3:2 TFA/TIPS/H_2O (4x, ~30 min each) and the combined cleavage solutions were dried under vacuum. The identity of **1-C16** was confirmed by LC-ESI-MS analysis (Figure S1). This material was used without further purification and yield was not determined.

1-C18, 1-C20, 1-C22, and 1-C24 (General Procedure). A 3.0 mL fritted polypropylene syringe equipped with a capped hypodermic needle was loaded with dry resin **1** (50 mg). The resin was washed with 2.5 mL of CH_2Cl_2 (3x, 3-5 min per wash). Next, the 4-methyltrityl (Mtt) protecting group was removed by treatment with 2.5 mL of 94:5:1 CH_2Cl_2/TIPS/TFA at RT (5x, 5 min each) followed by washing with 2.5 mL of NMP (3x, 3-5 min per wash). The resin was then carefully transferred to a 1.5 mL microcentrifuge tube. The resin was treated with a solution of the appropriate carboxylic acid (R_2-COOH, 0.12 mmol), PyBOP (65 mg, 0.12 mmol), HOBt (16 mg, 0.12 mmol), and DIPEA (65 μL, 0.38 mmol) in 1.0 mL of NMP. The reaction was incubated at 50 ^oC for 5 h. The reaction was then centrifuged, and 0.5 mL of the supernatant was discarded. An additional 0.5 mL of NMP was then added followed again by centrifugation and removal of 0.5 mL of the supernatant. This process was repeated two additional times. The resin slurry was carefully transferred to a 3.0 mL fritted polypropylene syringe equipped with a capped hypodermic needle and washed with 2.5 mL of NMP (3x, 3-5 min per wash). Fmoc removal was achieved by treatment with 2.5 mL of 80:20 NMP/piperidine for 20 min at RT followed by washing with 2.5 mL of NMP (3x, 3-5 min per wash) and 2.5 mL of CH_2Cl_2 (3x, 3-5 min per wash). The peptide was cleaved from the resin with 2.5 mL of 95:3:2 TFA/TIPS/H_2O (3x, ~30 min each) and the combined cleavage solutions were concentrated *in vacuo*. **1-C18**, **1-C20**, **1-C22**, and **1-C24** were precipitated from cold

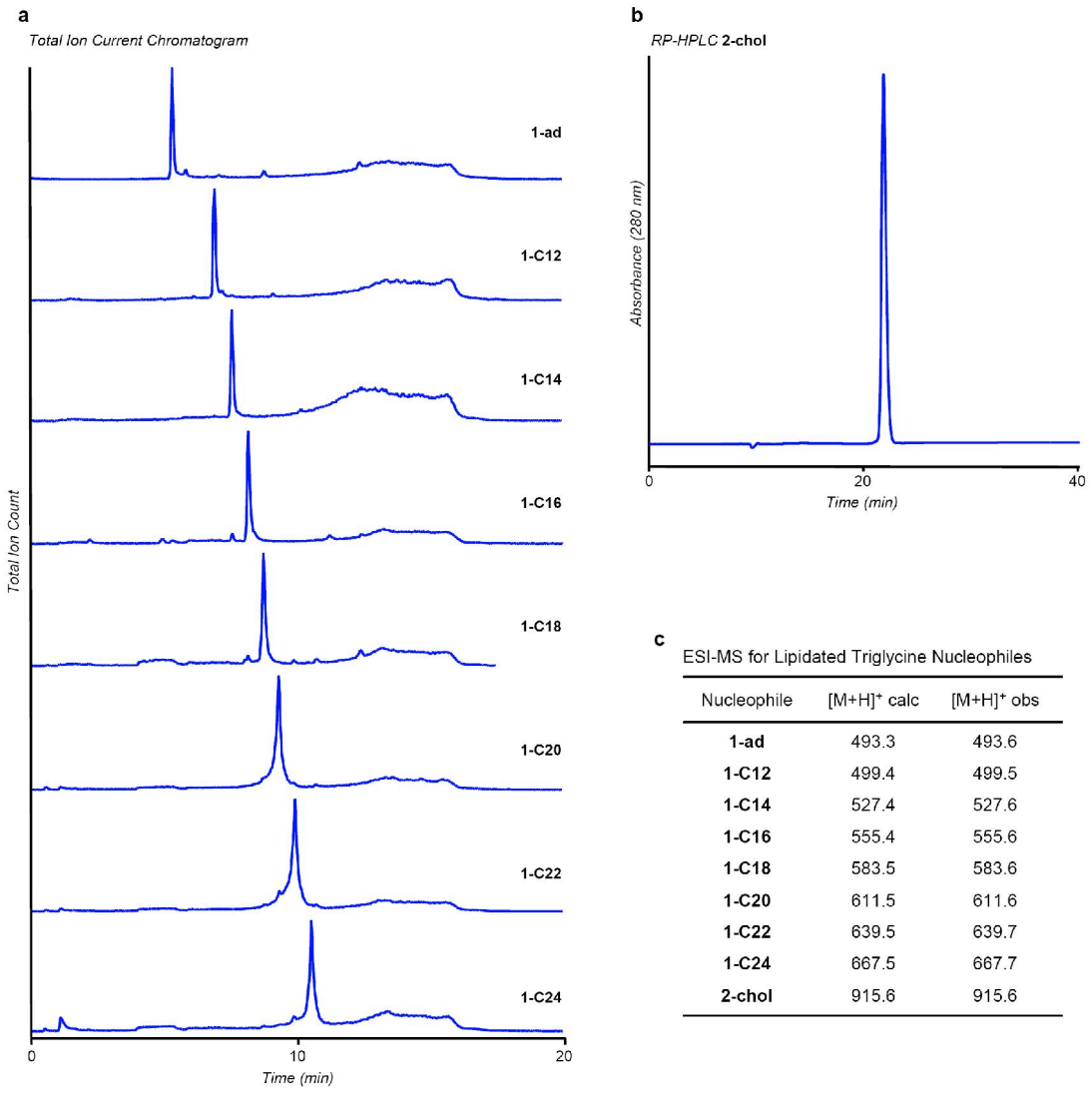
ether and dried under vacuum. The identity of all peptides was confirmed by LC-ESI-MS analysis (Figure S1). Peptides were used without further purification and yield was not determined.

Cholesterol NHS-carbonate (3). An oven dried round bottom flask equipped with an argon inlet was charged with cholesterol (500 mg, 1.29 mmol) and *N,N′*-disuccinimidyl carbonate (DSC) (665 mg, 2.59 mmol) in 15 mL of 1:1:1 CH_2Cl_2/MeCN/DIPEA (all anhydrous). The reaction was stirred at RT for 24 h. The reaction mixture was then partitioned between brine and CH_2Cl_2. The aqueous layer was extracted two additional times with CH_2Cl_2. The combined organic layers were then dried over $MgSO_4$, filtered, and concentrated. The remaining residue was then purified by flash chromatography (95:5 CH_2Cl_2/EtOAc) to yield **3** as a white solid (274 mg, 40%). ^{1}H NMR (400 MHz, $CDCl_3$): δ, 5.39 (d, 1H, J = 4.4 Hz), 4.57 (m, 1H), 2.81 (s, 4H), 2.46 (m, 2H), 2.05-0.80 (m, 26H), 1.00 (s, 3H), 0.89 (d, 3H, J = 6.4 Hz), 0.84 (d, 3H, J = 6.4 Hz), 0.83 (d, 3H, J = 6.8 Hz), 0.65 (s, 3H) . ^{13}C NMR (100 MHz, $CDCl_3$): δ, 169.0, 151.0, 138.7, 123.9, 82.4, 56.8, 56.3, 50.1, 42.5, 39.9, 39.7, 37.8, 36.9, 36.7, 36.4, 36.0, 32.1, 32.0, 28.4, 28.2, 27.6, 25.7, 24.5, 24.0, 23.0, 22.8, 21.2, 19.4, 18.9, 12.0. HRMS (ESI+) calculated for $C_{32}H_{49}NO_5Na$ ($[M+Na]^+$) 550.3503, found 550.3383.

Fmoc-Gly-Gly-Gly-Trp-Lys-CONH$_2$ (2). Intermediate **2** was synthesized on Rink amide resin following the procedure described above for the preparation of **1**. Peptide **2** was cleaved from the resin with 95:3:2 TFA/TIPS/H_2O (3x, ~30 min each). The combined cleavage solutions were concentrated and **2** was precipitated from ether and dried. The identity of **2** was confirmed by LC-ESI-MS. Yield not determined. LRMS (ESI+) calculated for $C_{38}H_{45}N_8O_7$ ($[M+H]^+$) 725.3, found 725.4.

2-chol. To a 1.5 mL microcentrifuge tube was added **3** (10.5 mg dissolved in 50 μL of $CHCl_3$, 19.9 μmol), **2** (7.2 mg dissolved in 50 μL of NMP, 9.9 μmol)**,** and anhydrous DIPEA (5.00 μL, 28.7 μmol). After overnight incubation at RT, piperidine (25 μL) was added and the reaction was incubated for an additional 2 h at RT. The reaction was then diluted with 0.5 mL of 2-propanol and fractionated by RP-HPLC [semi-preparative C18 column, 2-propanol:H_2O gradient mobile phase, 2 mL/min, 50% 2-propanol → 90% 2-

propanol (0-20 min)]. Fractions containing **2-chol** were pooled, concentrated, and further purified by RP-HPLC [semi-preparative PE column, 2-propanol:H_2O gradient mobile phase, 1 mL/min, 50% 2-propanol → 90% 2-propanol (0-20 min)] to yield **2-chol** (2 mg, 22%). The identity of **2-chol** was confirmed by LC-ESI-MS (Figure S1).



c

ESI-MS for Lipidated Triglycine Nucleophiles

Nucleophile	$[M+H]^+$ calc	$[M+H]^+$ obs
1-ad	493.3	493.6
1-C12	499.4	499.5
1-C14	527.4	527.6
1-C16	555.4	555.6
1-C18	583.5	583.6
1-C20	611.5	611.6
1-C22	639.5	639.7
1-C24	667.5	667.7
2-chol	915.6	915.6

Figure S1. Characterization of lipidated triglycine nucleophiles. (**a**) LC-ESI-MS total ion current chromatograms for lipidated triglycine nucleophiles. (**b**) RP-HPLC chromatogram (280 nm) for purified **2-chol**. (**c**) ESI-MS data for lipidated triglycine nucleophiles.

III. Protein Cloning and Expression

Sortase A Expression. Soluble sortase A bearing an N-terminal His_6-tag was expressed and purified from *E. coli* as previously described.[1]

eGFP-LPETG-His_6 cloning and expression. The gene encoding eGFP was PCR amplified from the commercially available pEGFP-N1 plasmid (Clontech) using forward primer 5'-CGCGCGCCATGGTGAGCAAGGGCGAGGAG-3' and reverse primer 5'-CGCGCGGATCCCGACCAGTTTCAGGAAGCTTGTACAGCTCGTCCATGCCG-3'. The PCR product was digested with NcoI and BamHI and ligated into pET28a+ (Novagen). This plasmid was then transformed into *E. coli* BL-21. In a typical experiment, cells were grown in two 2 L batches of sterile LB containing kanamycin (30 μg/mL) to an optical density of ~0.6-0.9 at 600 nm. Cells were induced with IPTG (1 mM) for 3 h at 37 ^{o}C. Cells were harvested by centrifugation and the pellet was stored overnight at -20 ^{o}C. The pellet was thawed and resuspended in 60 mL of 10 mM Tris pH 8.0, 100 mM phosphate, 300 mM NaCl, and 20 mM imidazole containing a protease inhibitor cocktail (complete mini tablet, EDTA-free, Roche) and treated with 240 μL of DNAse I (10 mg/mL in PBS), 480 μL of lysozyme (50 mg/mL in PBS), and 60 μL of $MgCl_2$ (1 M in PBS). The lysis reaction was incubated for 1 h at 4 ^{o}C. The cells were then sonicated and centrifuged to remove insoluble material. The pellet was then treated with an additional 20 mL of 10 mM Tris pH 8.0, 100 mM phosphate, 300 mM NaCl, and 20 mM imidazole, briefly sonicated, and centrifuged. The combined supernatants were applied to a Ni-NTA column consisting of 7.5 mL of commercial Ni-NTA slurry (Qiagen) equilibrated with 10 mM Tris pH 8.0, 100 mM phosphate, 300 mM NaCl, and 20 mM imidazole. The column was washed with three 45 mL portion of 10 mM Tris pH 8.0, 100 mM phosphate, 300 mM NaCl, and 20 mM imidazole. eGFP-LPETG-His_6 was eluted with ~10 mL of 10 mM Tris pH 8.0, 100 mM phosphate, 300 mM NaCl, and 300 mM imidazole. This material was concentrated and further purified by size exclusion chromatography on a HiLoad 16/60 Superdex 75 column (Amersham), eluting with 20 mM Tris pH 8.0, 150 mM NaCl at a flow rate of 1 mL/min. Fractions containing eGFP-LPETG-His_6 were pooled, concentrated, and stored at -80 ^{o}C.

Immediately prior to sortase-catalyzed transpeptidation, the eGFP-LPETG-His_6 stock was thawed and again purified by affinity chromatography over commercial Ni-NTA resin. After binding eGFP-LPETG-His_6 to the resin, the column was washed with three portions of 20 mM Tris pH 8.0, 150 mM NaCl, and 20 mM imidazole. The protein was eluted with 20 mM Tris pH 8.0, 150 mM NaCl, and 300 mM imidazole. This material was buffer exchanged into 20 mM Tris pH 8.0, 150 mM NaCl using a NAP™ 5 Sephadex™ column (GE Healthcare) and concentrated. The concentration was estimated by UV-Vis spectroscopy using the absorbance of eGFP at 488 nm (extinction coefficient 55,900 $M^{-1}cm^{-1}$).[2]

IV. Sortase-Mediated Protein Labeling and Characterization of Protein Conjugates

Ni-NTA slurry for removal of His_6-tagged proteins after transpeptidation. Removal of His_6-tagged proteins following transpeptidation was achieved with Ni-NTA resin treated in the following way: 500 μL of commercial Ni-NTA resin (Qiagen) was treated with 1 mL of 40 mM Tris pH 8.0, 1 M NaCl, and 40 mM imidazole. The mixture was then centrifuged and the supernatant was removed. This process was repeated two additional times. The resin was then suspended to a final volume of 500 μL with 40 mM Tris pH 8.0, 1 M NaCl, and 40 mM imidazole. This slurry was used to treat samples depicted in Figure 1a lanes 3-11 and Figure 2b lanes 3-13. For control experiments (Figure 2b, lanes 1-2), the above procedure was performed on a separate batch of commercial Ni-NTA resin using 40 mM Tris pH 8.0, 1 M NaCl, and 600 mM imidazole to block binding of His_6-tagged proteins.

Lipid modification of GFP-LPETG-His_6 and removal of His_6-tagged proteins (General Procedure). Lipidated triglycine nucleophile (0.5 μL of a 100 mM solution in DMSO) was combined with 2.5 μL of 10x sortase reaction buffer (500 mM Tris pH 7.5, 100 mM $CaCl_2$, 1.5 M NaCl) and 1.25 μL of 20% (w/v) *n*-dodecyl maltoside. This mixture was repeatedly heated in a 90 ^oC heat block and incubated in a sonicating water bath to disperse and solubilize the lipidated nucleophile. After cooling to room

temperature, eGFP-LPETG-His_6 (12.4 μL of a 110 μM solution in 20 mM Tris pH 8.0, 150 mM NaCl) and sortase A (8.35 μL of a 448 μM solution in 50 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol (v/v)) were added. The reaction mixtures were incubated at 37 ^{o}C for 5 h in the dark and then treated with 50 μL of the appropriate Ni-NTA slurry described above and incubated for an additional 2 h at room temperature. The mixtures were then filtered (Spin-X® Centrifugal Tube Filters 0.22 μm, Corning) to yield solutions containing lipid-modified eGFP. Samples were analyzed by SDS-PAGE with visualization by Coomassie blue staining.

Acetone Precipitation of eGFP conjugates and Characterization by ESI-MS. 20 μL of lipid-modified eGFP solution was treated with 1 mL of acetone and centrifuged at maximum speed for 3 minutes. The supernatant was removed and the pellet was washed with 1 mL of acetone. The sample was again centrifuged and the pellet was dissolved in 100 μL of 0.1% formic acid. Samples were then analyzed by LC-ESI-MS (Figure S2a).

MALDI-TOF MS Characterization of eGFP-2-chol. eGFP-**2-chol** was desalted by RP-HPLC [C4 column, MeCN:H_2O gradient mobile phase with 0.1% TFA, 1 mL/min, 5% MeCN $\rightarrow$ 50% MeCN (0-15 min), 50% MeCN $\rightarrow$ 80% MeCN (15-18 min)]. The fraction containing eGFP-**2-chol** was analyzed by MALDI-TOF-MS (Figure S2b).

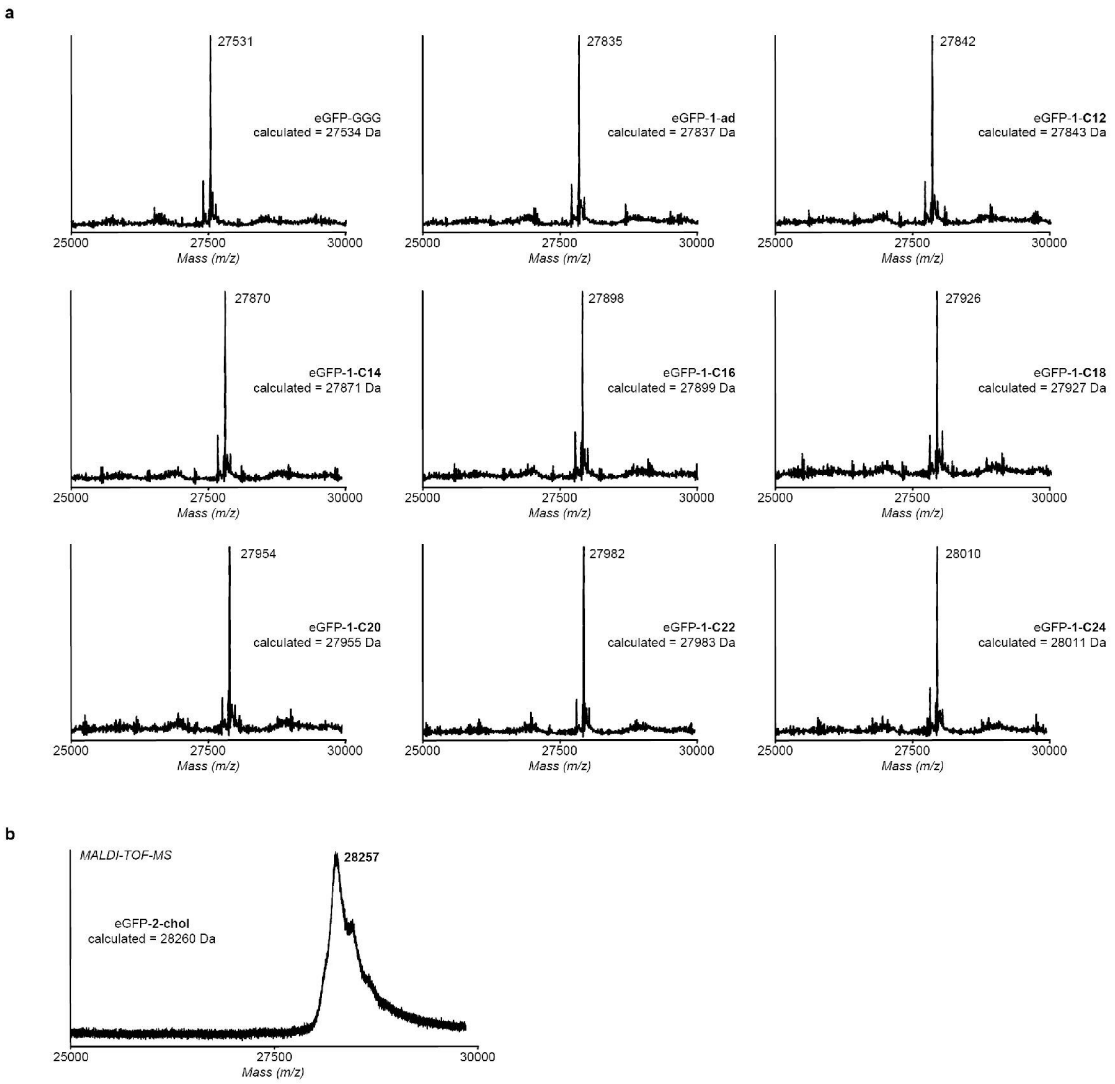


Figure S2. (**a**) Reconstructed ESI-MS spectra for modified eGFP. (**b**) MALDI-TOF MS spectrum of eGFP-**2-chol**.

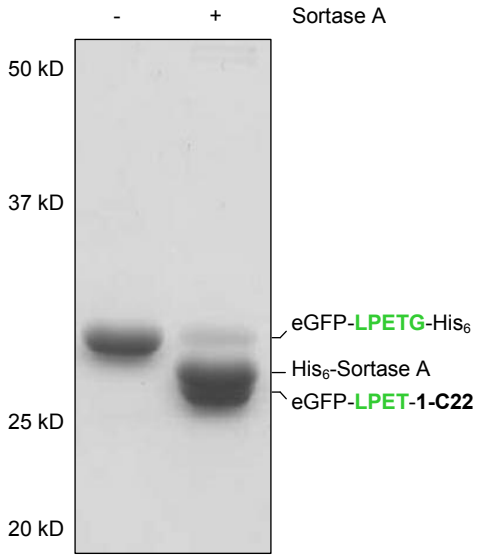
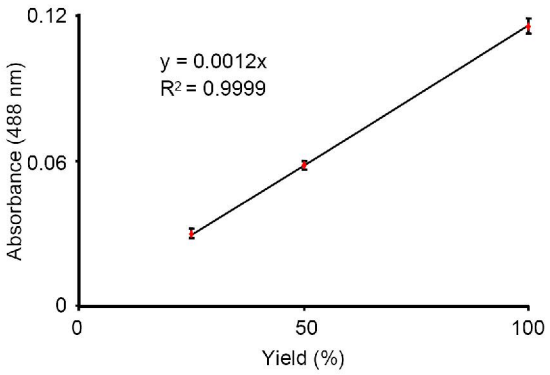


Figure S3. Negative control experiment demonstrating that the formation of the eGFP-LPETG-**1-C22** transpeptidation product requires sortase. Conditions: 77 μM eGFP-LPETG-His_6, 150 μM sortase A, 2 mM nucleophile, 1% (w/v) *n*-dodecyl maltoside, 50 mM Tris pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 5 h at 37 ^oC. Samples analyzed by SDS-PAGE.

Estimation of % Yield of eGFP conjugates. The yield of lipid modified eGFP was estimated using the absorbance of eGFP at 488 nm. As a standard, a sample of unmodified eGFP-LPETG-His_6 was incubated in the absence of sortase A and lipidated nucleophile (Figure 2b, lane 1) and treated with Ni-NTA resin containing excess imidazole to prevent protein binding. After filtration to remove Ni-NTA, the absorbance of this sample at 488 nm was measured and assumed to represent 100% recovery. Serial dilutions of this sample were measured in triplicate and used to construct a standard curve (Figure S4).

a



b

eGFP Conjugate	absorbance (488 nm)	yield (%)
eGFP-GGG	0.112	93
eGFP-**1-ad**	0.104	87
eGFP-**1-C12**	0.104	87
eGFP-**1-C14**	0.086	72
eGFP-**1-C16**	0.077	64
eGFP-**1-C18**	0.069	57
eGFP-**1-C20**	0.079	66
eGFP-**1-C22**	0.100	83
eGFP-**1-C24**	0.101	84
eGFP-**2-chol**	0.105	87

Figure S4. (**a**) Standard curve for estimating the yield of lipid-modified eGFP. (**b**) Absorbance of lipid-modified eGFP and estimated yield.

V. Cell Membrane Association of Lipid-Modified eGFP

1000x stock solutions of lipid-modified eGFP. Solutions of lipid-modified eGFP obtained by transpeptidation and depletion of His_6-tagged proteins were used without further purification for cell membrane association studies. Prior to addition to cells, the concentration of eGFP in each sample was measured by UV-Vis (488 nm), and all solutions were adjusted to a final eGFP concentration of 2.5 mg/mL. Dilutions were made with a mock reaction mixture lacking eGFP, sortase A, and lipidated triglycine nucleophile to ensure that all samples contained the same amount of residual components (*n*-dodecyl maltoside, imidazole, $CaCl_2$, etc.) from the sortase labeling procedure.

Cell culture and membrane association of lipid-modified eGFP. HeLa cells were maintained in DME media supplemented with 10% fetal bovine serum (FBS), penicillin (50 units/mL), and streptomycin sulfate (50 μg/mL). Cells were incubated in a 5% CO_2 humidified incubator at 37 ^{o}C. One day prior to treatment with lipid-modified eGFP, cells were seeded into 6-well polystyrene cell culture plates at a density of ~100,000 cells per well and incubated overnight. The media was then removed and replaced with 2 mL of serum-free DME containing 2.5 μg/mL modified eGFP (lipid modified proteins were delivered as 1000x stock solutions, see above). Cells were then incubated for 1 h at 37 ^{o}C. Next, the cells were washed three times with ~4 mL of cold PBS containing 1% FBS. The cells were lifted from the culture plate by treatment with 1 mL of 1 mM EDTA in PBS and pelleted at 250 x g for 5 min. The cells were then resuspended in 200 μL of PBS containing 1% FBS and propidium iodide (0.5 μM) and analyzed by flow cytometry.

Control experiment of eGFP-GGG in combination with lipidated triglycine nucleophile. Cells were seeded into 6-well polystyrene cell culture plates at a density of ~100,000 cells per well and incubated overnight. The media was then removed and replaced with 2 mL of serum-free DME containing 2.5 μg/mL eGFP-GGG and 1 μM lipidated triglycine nucleophile (**1-C22**, **1-C24**, or **2-chol**). Cells were incubated for 1 h at 37 ^{o}C. Next, the cells were washed three times with ~4 mL of cold PBS containing 1% FBS. The cells were lifted from the culture plate by treatment with 1 mL of 1 mM EDTA

in PBS and pelleted at 250 x g for 5 min. The cells were then resuspended in 200 µL of PBS containing 1% FBS and propidium iodide (0.5 µM) and analyzed by flow cytometry (Figure S5).

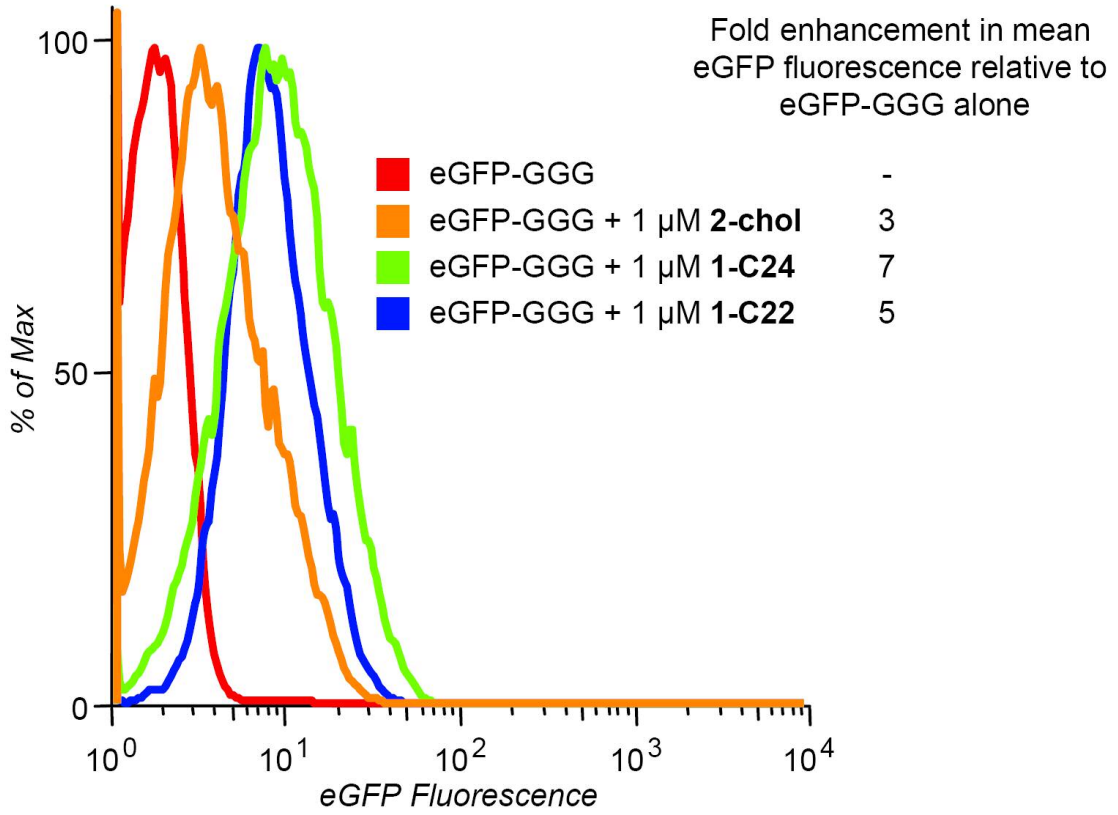


Figure S5. Control experiment demonstrating minor increases in cellular fluorescence as a result of incubation with eGFP-GGG in combination with free lipidated triglycine nucleophiles.

Spinning Disc Confocal Microscopy. HeLa and U373 cells were maintained in DME media supplemented with 10% fetal bovine serum (FBS), penicillin (50 units/mL), and streptomycin sulfate (50 µg/mL). Cells were incubated in a 5% CO_2 humidified incubator at 37 ^{o}C. One day prior to treatment with lipid-modified eGFP, cells were seeded into Lab-Tek^{TM} II chambered coverglass slides (Nalge Nunc International) and allowed to adhere overnight. The media was then removed and replaced with 200 µL of serum-free DME containing 2.5 µg/mL modified eGFP (lipid modified proteins were

delivered as 1000x stock solutions, see above). Cells were then incubated for 1-5 h at 37 ^{o}C. Next, the cells were washed three times with ~0.5 mL of cold PBS containing 1% FBS. Cells were then covered with 200 uL of PBS containing 1% FBS and imaged. For transferrin colocalization, U373 were incubated with 200 μL of serum-free DME containing 2.5 μg/mL modified eGFP for 5 h at 37 ^{o}C. Transferrin-Alexa 647 (Invitrogen, 100 μg/mL final concentration) was added during the final 15 minutes of this incubation. Cells were then washed as described above and imaged.

References

1. Ton-That, H.; Liu, G.; Mazmanian, S. K.; Faull, K. F.; Schneewind, O. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 12424-12429.

2. Tsien, R. Y. *Annual Review of Biochemistry* **1998**, *67*, 509-544.